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The relevance of mycorrhizosphere on the antimicrobial activity mediated by *Streptomyces* spp. interactions

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Abstract

The emergence of antimicrobial drug resistance has stimulated the scientific community to find efficient and faster ways to search for new antimicrobial compounds. To this end, the development of new approaches, as the microbial co-culture, employing ecological knowledge of microbial community dynamics is of great interest. In soils, antibiotic production seems to be tightly regulated by social interactions among *Streptomyces* species, which in turn are involved in a wider network of inter-kingdom interactions, including with plant roots and mycorrhizal fungi in the mycorrhizosphere. The aim of this study was to investigate whether mycorrhizosphere context (arbuscular mycorrhizal fungi, AMF, colonizing the host plant rhizosphere) influences *Streptomyces* antagonistic phenotypes assembly and antimicrobial activity mediated by *Streptomyces* spp. pairwise interactions. Moreover, it was evaluated if genetic relatedness among *Streptomyces* spp. is an important determinant in inducing changes in antimicrobial activity during pairwise interactions.

To accomplish these goals, several *Streptomyces* strains were isolated from distinct maize plant mycorrhizosphere communities, which were previously manipulated by the addition of different AMF species inocula thereby creating different AMF communities. Their antimicrobial activity in monoculture and in pairwise interactions was tested against different target bacterial species.

The results from the monoculture assays showed no differences between AMF treatments in the frequency and intensity of inhibition, suggesting that AMF community composition associated with the plant rhizosphere did not influence the selection of *Streptomyces* spp. antagonistic phenotypes. In contrast, results from pairwise interactions showed a mycorrhizosphere influence on shifting antimicrobial activity of *Streptomyces* strains when grown in the presence of a partner. The frequency of changes in antimicrobial activity during *Streptomyces* pairwise interactions was significantly higher among strains isolated from the same than from different mycorrhizosphere origins, with emphasis on the occurrence of “new” inhibitions that were not observed in monoculture. Furthermore, the frequency and type of changes in inhibitory capacity of *Streptomyces* strains varied among different AMF communities.

Genetic distance results indicated that changes in antimicrobial activity were more likely to occur among more closely related *Streptomyces* strains.

Results of this study indicate that mycorrhizal context of the rhizosphere is an important factor influencing the outcome of *Streptomyces* spp. interactions in antimicrobial activity, and give support to the theory that applying ecological concepts and understanding the microbial community dynamics to studies fostering new drugs can help to choose the best co-culture partners.

Keywords: Antimicrobial activity changes, arbuscular mycorrhizal fungi, microbial interactions, mycorrhizosphere, *Streptomyces* spp.

Resumo

O aumento de estirpes patogénicas resistentes aos compostos antimicrobianos comercializados, quer na área da saúde (Martinez *et al*., 2009) quer na área agrícola (Palaniyandi *et al*., 2013), tem sido foco de atenção da comunidade científica nos últimos anos. Esta problemática tem incentivado o desenvolvimento de estratégias rápidas e eficientes para a descoberta de novos compostos com atividade antimicrobiana. A co-cultura de microrganismos em laboratório é uma das estratégias que tem vindo a ser explorada, consistindo no crescimento em culturas mistas de duas ou mais estirpes microbianas com o intuito de promover interações entre os microrganismos que possam levar à ativação de vias biossintéticas silenciadas (Antoraz *et al*., 2015). Na natureza os microrganismos encontram-se em comunidades, onde estabelecem inúmeras interações entre si. Neste sentido, tem-se procurado tirar partido de conhecimentos da dinâmica das comunidades para o desenvolvimento de novas abordagens para desbloquear vias de síntese de antibióticos que estão silenciadas ou que são desconhecidas (Smanski *et al*., 2016).

O género *Streptomyces* é um grupo bacteriano conhecido pelo seu vasto leque de metabolitos secundários, em particular pela produção de mais de dois terços dos antibióticos que são comercializados (Bérdy, 2012). Cerca de 5 a 10% do seu genoma está associado a vias biossintéticas que codificam a produção de antibióticos, no entanto muitas destas vias não são expressas constitutivamente quando as estirpes são crescidas em condições laboratoriais usualmente utilizadas (Cornforth e Foster, 2013). Assim, a aplicação de novas abordagens (em particular a co-cultura) em estudos para a descoberta de novos antibióticos em bactérias do género *Streptomyces* é de grande interesse.

O género *Streptomyces* está muito representado na rizosfera, zona do solo que envolve as raízes das plantas. No entanto, uma vez que a maioria das plantas terrestres estabelece relações simbióticas com fungos micorrízicos arbusculares (FMA) (Veresoglou *et al*., 2012), o termo que melhor descreve esta zona do solo é micorrizosfera, pois é influenciada tanto pelas raízes como pelo fungo micorrízico (Johansson *et al*., 2004). As plantas exudam uma vasta variedade de moléculas através das raízes alterando o ambiente químico do solo. A quantidade e composição destes

exudados radiculares influenciam a estrutura das comunidades microbianas, uma vez que os microrganismos utilizam estes compostos no seu crescimento (ex. Haichar *et al*., 2008; Bakker *et al*., 2013a; Kaiser *et al*., 2014). A densidade e a presença de diferentes grupos funcionais bacterianos (Johansson *et al*., 2004), incluindo as populações de *Streptomyces* (Frey-Klett *et al*., 2007), podem ser influenciadas pela identidade de FMA em simbiose com as plantas, pois diferentes associações planta-FMA podem levar a padrões de exudação distintos criando assim distintos *pools* de nutrientes (Drigo *et al*., 2010). Bactérias do género *Streptomyces*, e os microrganismos no geral, encontram-se em comunidades, onde partilham os mesmos nichos ecológicos e por isso a comunicação química entre os microrganismos parece ser de grande relevância para a sua sobrevivência (Raaijmakers e Mazzola, 2012). De facto, em *Streptomyces* a produção de antibióticos assim como a sua supressão parecem ser intimamente reguladas por interações sociais que se estabelecem entre as estirpes (Jauri e Kinkel, 2014; Abrudan *et al*., 2015). Recentemente, verificou-se que no solo a seleção de estirpes com atividade antimicrobiana varia com o local de onde foram isoladas, presumivelmente porque em diferentes locais as dinâmicas das comunidades são diferentes e as interações de competição e/ ou cooperação contribuem diferentemente para o *fitness* bacteriano (Kinkel *et al*., 2014). Um outro estudo, ao investigar interações entre estirpes de *Streptomyces,* observou uma maior frequência de alterações nos fenótipos inibitórios assim como uma maior intensidade de inibição quando se utilizou estirpes isoladas do mesmo local em comparação com interações entre estirpes isoladas de locais distintos (Jauri e Kinkel, 2014). Assim, nos estudos para a pesquisa de novos antibióticos parece ser da maior importância aliar os conhecimentos das dinâmicas das comunidades microbianas bem como ter em conta o contexto ecológico das estirpes selecionadas.

Este trabalho teve como objetivo investigar a influência do contexto biótico da micorrizosfera na seleção de estirpes de *Streptomyces* com fenótipos antagonistas assim como nas alterações da atividade antimicrobiana durante as interações entre estirpes deste género. Neste sentido, foram isoladas 50 estirpes de *Streptomyces* da rizosfera de plantas de milho de cinco tratamentos diferindo na comunidade de FMA associada à rizosfera, que foram previamente manipuladas pela introdução de inóculos de diferentes espécies de fungos micorrízicos (4 espécies diferentes e um tratamento onde não ocorreu adição de inóculo) para averiguar: i) se a composição das comunidades de

FMA tem influência na seleção de *Streptomyces* spp. com fenótipos antagonistas; ii) se a frequência e o tipo de alterações (aumento ou diminuição) na atividade antimicrobiana induzida por interações está dependente da origem micorrizosférica das estirpes (isto é, co-cultura entre duas estirpes isoladas da mesma origem ou tratamento de FMA *vs.* de diferentes origens) e se está dependente da comunidade de FMA presente em cada tratamento de onde foram isoladas; e por último iii) se a distância genética entre as estirpes é determinante nas alterações da atividade antimicrobiana. Para responder aos objetivos do trabalho, foi avaliada a atividade inibitória das estirpes de *Streptomyces* em monocultura, contra estirpes bacterianas alvo, duas Gram positivas (*Bacillus megaterium* e *Clavibacter michiganensis*) e uma Gram negativa (*Pseudomonas syringae*), e em co-cultura (contra as mesma bactérias alvo). Nos ensaios de co-cultura foram realizadas interações de dois tipos: 1) interações entre pares de estirpes de *Streptomyces* isoladas da mesma origem micorrizosférica, isto é, isoladas do mesmo tratamento de FMA, e 2) interações entre estirpes isoladas de origens diferentes. As alterações na atividade antimicrobiana foram avaliadas comparando as zonas de inibição do crescimento das bactérias alvo em co-cultura com as zonas de inibição em monocultura, e classificadas em aumento (intensificação ou "nova" inibição) ou diminuição (atenuação ou supressão) da atividade antimicrobiana. Para cada interação, foi também determinada distância genética entre as estirpes de *Streptomyces*.

Os resultados deste trabalho mostraram que as diferentes comunidades de FMA nos cinco tratamentos não levaram a uma seleção diferencial de estirpes de *Streptomyces* com fenótipos antagonistas, nem em termos de frequência de inibidores nem de intensidade de inibição. Por outro lado, revelaram que há maior frequência de alterações na atividade inibitória em interações entre estirpes de *Streptomyces* isoladas da mesma origem micorrizosférica (45%) do que quando as estirpes em interação foram isoladas de origens diferentes (33%). É de realçar que a maior frequência de alterações entre estirpes da mesma origem micorrizosférica se deveu maioritariamente a casos em que houve aumento da atividade antimicrobiana, mais especificamente a uma maior ocorrência de "novas" inibições, isto é, duas estirpes em co-cultura inibiram uma bactéria alvo que não era inibida por nenhuma das duas estirpes de *Streptomyces* quando em monocultura. Os resultados mostraram ainda que a comunidade de FMA influenciou o número total de alterações na atividade antimicrobiana, assim como

alguns tipos de alterações (“novas” inibições e atenuações). Os resultados revelaram também que a proximidade genética das estirpes em co-cultura é um fator determinante na ocorrência de alterações na atividade antimicrobiana.

O presente estudo realçou não só o potencial da utilização da co-cultura como técnica para a deteção de novos compostos antimicrobianos como revelou ainda que a micorrizosfera é um importante *“hotspot”* para a seleção de potenciais parceiros nesses ensaios de co-cultura. O interesse no estudo das interações sociais entre os microrganismos e as dinâmicas das suas comunidades tem vindo a aumentar. De facto, aplicar esses conhecimentos ecológicos na seleção de estirpes a utilizar nos ensaios de co-cultura, pode revelar-se uma mais-valia para a descoberta de uma forma mais rápida e eficiente de novos compostos com atividade antimicrobiana.

Palavras-chave: Alterações de fenótipos inibitórios, atividade antimicrobiana, fungos micorrízicos arbusculares, interações microbianas, micorrizosfera, *Streptomyces* spp.

Index

1 – Introduction

The emergence of antimicrobial drug resistance either among clinical and agricultural pathogens has stimulated the scientific community to find efficient and faster ways to search for new antimicrobial compounds. The genus *Streptomyces* is ubiquitous in rhizosphere soils (Barka *et al*., 2016), and is a recognized source of natural products such as antibiotics. In fact, most of known antibiotics are produced by members of this genus, but only a tiny portion of the repertoire of bioactive compounds has been isolated since some of the bioactive pathways are not expressed in standard laboratory conditions (Zhu *et al*., 2014; Antoraz *et al*., 2015). Nowadays, the challenge is to explore the unknown biosynthetic pathways to the discovery of new drugs. To this end, it is of great interest to integrate ecological knowledge of microbial community dynamics on the developing of new approaches for antimicrobial compounds discovery in microbes, particularly from the genus *Streptomyces* (Smanski *et al*., 2016).

1.1 – General characteristics of the genus *Streptomyces*

The genus *Streptomyces* belongs to the phylum *Actinobacteria*, a group of Gram positive bacteria characterized by a high percentage of guanine-cytosine content in its genome (Anderson and Wellington, 2001). Bacteria of this genus are commonly found in soil and rhizosphere, playing an important role in soil nutrient dynamics due to the production of a vast variety of extracellular enzymes. Such extracellular activity enables them to participate in the degradation of soil organic matter and to play a role in nutrient cycling by hydrolyzing complex polymers such as cellulose, lignin and chitin (Barka *et al*., 2016). In this way, they have the capability to colonize multiple niches, having an important role regulating soil microbial communities (Procópio *et al*., 2012). Species of the genus *Streptomyces* could act as plant growth promoting-rhizobacteria (PGPR), for example by producing the plant hormone indole-3-acetic acid (Doumbou *et al*., 2002). It is also well documented the suppression of plant diseases by several *Streptomyces* strains through antagonistic interactions against diverse plant pathogenic microbes (Bressan and Figueiredo, 2008; Hiltunen *et al*., 2009), thereby showing their potential as biocontrol agents.

Members of this genus have particular characteristics such as their complex morphology as a filamentous substrate mycelium which leads to a network of hyphae by their apical growth and lateral branching (Figure 1).

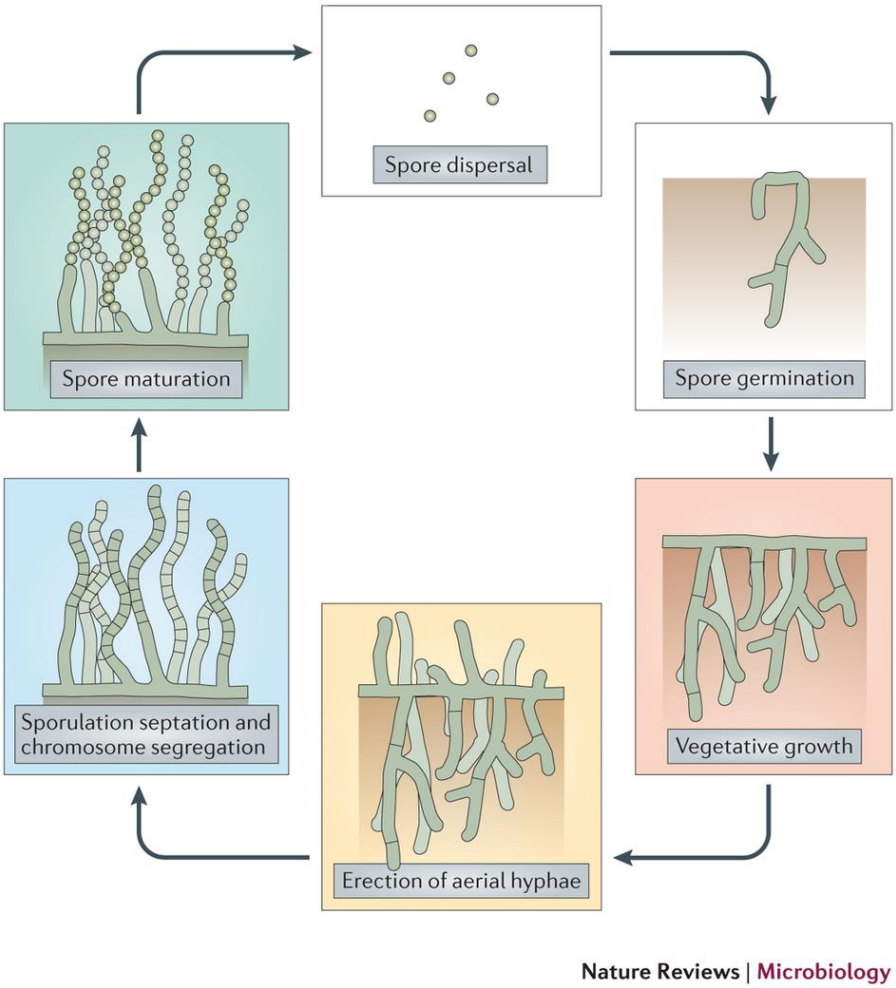

Figure 1: Schematic representation of *Streptomyces* life cycle. Spores produce germ tubes to develop the vegetative mycelium which grow by tip extension and branching. The aerial mycelium emerge from the colony surface into the air developing specialized reproductive structures where differentiation of spores occur. After maturation spores are dispersed (Scheme credit to Bush *et al.*, 2015).

This vegetative mycelium has nutritional functions (Bush *et al.*, 2015). In some conditions, such as nutrient limitation, colony age or other stress conditions, the substrate mycelium undergoes a programmed cell death-like mechanism providing nutrients for the formation of aerial mycelium, which is subsequently differentiated into chain of spores (Flärdh and Buttner, 2009; Bush *et al.*, 2015). This process could be coupled with the production of secondary metabolites. The genus *Streptomyces* is also known for its genetic complexity. The genome is arranged in one large, linear

chromosome with the presence of linear or circular plasmids. *Streptomyces* spp. have a remarkable percentage of secondary metabolite gene clusters and a huge number of components in genome dedicated to the regulation of secondary metabolic pathways (Zhou *et al*., 2012). The expression of these biosynthetic pathways in *Streptomyces* spp. could be influenced by many factors, including extracellular signals, the type of carbon and nitrogen sources as well as the availability of these compounds (Manteca *et al*., 2008). The secondary metabolism of *Streptomyces* spp. has a great interest due to the production of high-value commercial compounds such as antibiotics, antifungals and likewise other biomolecules with pharmaceutical relevance like compounds with antitumor or immunosuppressive properties (Challis and Hopwood, 2003) as well as molecules with agriculture importance as growth promoters and plant disease protectors (Prapagdee *et al*., 2008) and with industrial applications as extracellular enzymes (Tuncer *et al*., 2004).

Among the many functions of *Streptomyces* spp., antimicrobial compounds production with emphasis on antibiotics, is of great interest for medicine and agriculture and also for the understanding of microbial interactions in nature.

1.2 – Strategies to explore *Streptomyces* spp. production of new antimicrobial compounds: The co-culture approach

The extensive use and misuse of antimicrobial compounds, either in clinical (Martinez *et al*., 2009) as in agricultural practices (Palaniyandi *et al*., 2013), has been rising the resistance of pathogenic strains to commercial drugs. Therefore, the search for new antimicrobial compounds is crucial. The genus *Streptomyces* is a well-recognized source of antimicrobial compounds, producing more than two thirds of naturally occurring antibiotics (Bérdy, 2012). About 5-10% of a *Streptomyces* genome is associated with antibiotic biosynthetic pathways. However, part of these biosynthetic pathways encoding for those compounds are not expressed under standard laboratory conditions, and even in nature some antimicrobial compounds may be produced only in response either to environmental signals or to those produced by neighboring microorganisms, since some of these pathways do not have a constitutive expression (Cornforth and Foster, 2013). In this way, new approaches designed to reveal unknown or awake silent

biosynthetic pathways in *Streptomyces* have taken a particular interest to discover new inhibitory molecules.

One approach comprises the isolation of species from unexplored environments (Zhang and Moore, 2015). Another approach is the development of *in situ* cultures techniques (Antoraz *et al*., 2015), that is, the cultivation of microorganisms in their natural habitats. This new method is a powerful tool to explore the potential of microorganisms that were considered as unculturable, and thus to study new biosynthetic pathways.

Improved culture media by nutrient supply is also a new technique to elicit the production of antimicrobial compounds since it is known that nutritional signals affect regulatory cascades of antibiotic production pathways (Antoraz *et al*., 2015). For instance, it was demonstrated that compounds like phosphate or with nitrogen and iron act as signals triggering the activation of different two-component systems in *Streptomyces* spp. leading to differential expression of antibiotic gene clusters (Sola-Landa *et al*., 2003; Shu *et al*., 2009). Thus, adding nutrients commonly present in *Streptomyces* habitats, but that are not frequently used in laboratory culture media, could induce the expression of silent biosynthetic pathways.

In the same way, the addition of hormones and antibiotics to the culture media is a new strategy that has been adopted to stimulate the antibiotic production. Recent studies showed that antibiotics in low concentration, as well as certain hormones, can promote cell to cell communication leading to the activation of several cellular processes including secondary metabolites expression (Romero *et al*., 2011; Sidda and Corre, 2012).

Lastly, microorganism co-culture has recently emerged as a new tool to achieve the environmental scenario in the lab, since microorganisms in nature are found in multispecies communities where they communicate and thereby establish countless interactions (Antoraz *et al*., 2015). It has been shown that cultivation of two or three microorganisms in mix cultures is a successful way to discover new antimicrobial compounds (e.g. Marmann *et al*., 2014; Schroeckh *et al*., 2014; Netzker *et al*., 2015). Traxler *et al*. (2013) demonstrated that *Streptomyces coelicolor* produces different metabolites, many of them unidentified, depending on the partner species in co-culture, showing the huge plasticity in the expression of biosynthetic pathways of this species

and evidencing the potential of this co-culture approach to explore new antimicrobial compounds.

The production capacity of secondary metabolites is not uniformly found across microorganisms and environments. The scientific community has been investigating the ecological and evolutionary roles of antibiotics in natural interactions among microorganisms, and has been using that knowledge to drive the research efforts to identify hotspots and settings of new antimicrobial compounds (Smanski *et al*., 2016). Linking ecological concepts to the co-culture approach rather than using strain collections taken out of their ecological context would be of major relevance.

1.3 – Ecology of the genus *Streptomyces* in soil: The role of antimicrobial compounds in microbial interactions

Bacteria of the genus *Streptomyces* and microorganisms in general are found in soil in established communities that share the same ecological niches and where they need to develop strategies to survive (Raaijmakers and Mazzola, 2012). Therefore, chemical communication among microorganisms is crucial for self-defense.

The mechanisms of microbial communication have attracted much attention of the scientific community over the last years, specifically the role of antibiotics in nature and their impact mediating interactions among microbial populations have been largely discussed. Traditionally, antibiotics in soils are perceived as weapons that confer a fitness benefit to producers in competitive environments (Martinez *et al*., 2009; Antoraz *et al*., 2015). However, some authors have argued that antibiotic concentration in soil is too low to inhibit competitors (Raaijmakers and Mazzola, 2012). Thus, the perspective that they act as cooperative signals have been defended. It has been demonstrated that at sub-lethal concentration, antibiotics can induce shifts in microbial metabolism and change microbial behavior, for instance inducing biofilm formation or increasing motility (Linares *et al*., 2006; Ratcliff and Denison, 2011). Recently, the cooperative view has been challenged. Abrudan *et al*. (2015) showed that *Streptomyces* strains, in the presence of competitors from the same bacterial genus, increased their own inhibitory activity or suppressed antibiotic production of the competitors in order to have benefits in competitive social environments. This study reveals that social interactions have deep impacts on antibiotic production, and suggests that, at least under nutrient limited

conditions, antibiotics are predominantly used as weapons. Additionally, it has been demonstrated that depending on the location where *Streptomyces* species were isolated, the selection of *Streptomyces* species with antimicrobial activity varied, perhaps because competitive and cooperative interactions contribute differently to fitness benefits in distinct communities (Kinkel *et al*., 2014). Further, the frequency and intensity of antagonistic interactions among *Streptomyces* spp. is higher in within-community interactions (sympatric) than between communities (allopatric). One reasonable explanation is niche overlap within sympatric populations that can be linked to genetic relatedness among the individuals (Jauri and Kinkel, 2014; Kinkel *et al*., 2014). The rhizosphere is an environment where niche overlap can be high and where *Streptomyces* spp. are abundant.

The understanding of the role of antibiotics in soil continues to evolve, highlighting the potential of use of microbial interactions for the discovery of new antimicrobial compounds. Antibiotic production, or suppression, seems to be tightly regulated by social interactions among *Streptomyces* species, which in turn are involved in a wider network of inter-kingdom interactions in the rhizosphere.

1.4 – Plant-microbiome interactions: The influence of mycorrhizosphere on *Streptomyces* spp. antimicrobial activity

Plants have profound impacts shaping the soil microbiome. In particular, plant species or genotype is an important determinant in the selection of soil microbes (Berg and Smalla, 2009; Lundberg *et al*., 2012). There is evidence that the identity of host plant species can differentially influence the assembly and antagonistic potential of *Streptomyces* populations in rhizosphere soil (Bakker *et al*., 2013a, 2014).

It has been hypothesized that the plant-driven effect on associated antagonistic *Streptomyces* communities is mediated through changes in the chemical environment in soil (Bakker *et al*., 2013b). Plant roots exude a wide range of molecules into the rhizosphere, thereby altering soil chemical environment and providing nutrients for the microbial populations. The amount and composition of root exudates has the ability to influence microbial communities' structure (e.g. Haichar *et al*., 2008; Kinkel *et al*., 2012; Kaiser *et al*., 2015).

Though plant species identity is likely an important factor to take into account in the variation of production of antimicrobial compounds and in the diversity of antagonistic phenotypes, it seems reasonable to hypothesize that there are other rhizosphere biotic interactions within a given host plant species that can influence plant effects on *Streptomyces* antagonistic dynamics. The rhizosphere is the zone of soil that surrounds and is influenced by living roots. But, most of plants in terrestrial environment establish symbioses in the roots with arbuscular mycorrhizal fungi (AMF) (Veresoglou *et al*., 2012). These plant root-AMF associations have a crucial impact on rhizosphere and soil characteristics, thus the term which better describes this zone of soil is mycorrhizosphere, which is influenced by both roots and mycorrhizal fungi (Johansson *et al*., 2004).

The mycorrhizosphere is one of the most dynamic interfaces on Earth, where a myriad net of interactions between plants and microorganisms occur. The outcomes of these interactions have crucial impacts on microbial communities' structure, which in turn influence the growth, nutrition and health of plants (Philippot *et al*., 2013). Since there is no specificity on plant-AMF symbiosis, each host plant genotype can establish associations with different AMF taxa, leading to variation in the functional outcome of the symbiosis (Klironomos, 2003). The identity of fungi in plant root-AMF symbiosis can differently affect the density and occurrence of distinct microbial functional groups (Johansson *et al*., 2004), including *Streptomyces* populations (Frey-Klett *et al*., 2007), presumably because different plant root-AMF associations result in distinct exudation profiles leading to changes in the pool of available nutrients (Drigo *et al*., 2010). Therefore, individuals from the same host plant identity can have different AMF communities assembled in the roots forming different mycorrhizosphere communities. All these evidence suggest that the composition and/or diversity of AMF community might play a role in *Streptomyces* community antimicrobial activity in the rhizosphere.

1.5 – Objectives

Exploring the biotic context of the rhizosphere in which *Streptomyces* spp. live and socially interact can provide important insights in the novel co-culture approach for fostering new antimicrobial compounds.

The main goal of this work was to investigate whether mycorrhizosphere context (AMF associated with the host plant rhizosphere) influences *Streptomyces* antagonistic phenotypes assembly and antimicrobial activity mediated by pairwise interactions among *Streptomyces* spp. Specifically, several *Streptomyces* strains were isolated from distinct maize plant mycorrhizosphere communities, which were manipulated by the addition of different AMF species inocula creating different AMF communities, to respond to the following questions:

1) Does AMF community composition associated with the plant rhizosphere influence the selection of *Streptomyces* spp. antagonistic phenotypes?
2) Are frequency and type of changes (increase or decrease) in antimicrobial activity induced by pairwise interactions among *Streptomyces* spp. dependent on:
   2.1) Whether the strains are isolated from the same or different mycorrhizosphere origins (mycorrhizosphere origin effects)?
   2.2) The AMF community present in the rhizosphere (AMF community effects)?
3) Is genetic relatedness among *Streptomyces* spp. an important determinant in inducing changes in antimicrobial activity during pairwise interactions? And if so, does it explain potential mycorrhizosphere-context dependence effects on the occurrence of those changes?

To achieve these goals, antimicrobial activity of *Streptomyces* strains was evaluated in monoculture as proportion and intensity of inhibition in each mycorrhizosphere-community origin (AMF inoculation treatments). Further, *Streptomyces* spp. pairwise interaction outcomes were compared, in terms of proportion of changes in antimicrobial activity and direction of that changes (increase or decrease of antimicrobial activity in co-culture when comparing with inhibitory capacity in monoculture), in interactions within the same and across different mycorrhizosphere origins. Moreover, genetic relatedness among *Streptomyces* strains was also assessed.

It was expected that as a result of distinct AMF species additions, the environmental chemical characteristics of the rhizosphere were affected, and thus, the selection of antagonistic phenotypes varied among AMF treatments, particularly when compared to the absence of AMF addition, which represented the undisturbed environment. Also, the coexistence of *Streptomyces* strains in the same mycorrhizosphere, which may share the same niche (for example the utilization of the same nutrients), was expected to lead to more interactions within-mycorrhizosphere community resulting in changes in *Streptomyces* antimicrobial activity. Moreover, the outcome of antimicrobial activity mediated by interactions among *Streptomyces* spp. may be conditioned by different AMF communities (through AMF inocula addition). Further, it was expected that interactions among more closely related *Streptomyces* strains resulted in high proportion of changes in antimicrobial activity.

The intention of this work was not only to highlight the potential of co-culture approach to trigger the production of new antimicrobial compounds mediated by *Streptomyces* spp. interactions, but also to provide support to the theory that applying ecological concepts and the understanding of microbial community dynamics to studies fostering new drugs can help to choose the best co-culture partners.

2 – Materials and methods

2.1 – Isolation and growth conditions of *Streptomyces* strains

Streptomyces strains isolated in the present study were obtained from rhizosphere soil samples collected in a previous greenhouse experiment conducted at Centre for Ecology, Evolution and Environmental Changes (cE3c), University of Lisbon. In this experiment, different AMF communities were constructed by inoculum additions of single AMF species into a native soil AMF background community. Introduction of an AMF strain generally alters rhizosphere AMF community composition, diversity and/or species abundance (Mummey *et al*., 2009). Maize (*Zea mays* cv. Sincero) plants were grown in pots wherein a mixture of autoclaved sand and no autoclaved field soil collected from a maize crop field at Aveiro region, Portugal, was inoculated (approximately 3% w/w) with 1 of 4 distinct AMF species inocula, namely *Rhizoglomus intraradices*, *Funneliformis mosseae*, *Claroideoglomus claroideum*, or *Gigaspora* sp. A non AMF addition was also included (Figure 2). After 2 months, rhizosphere soil samples from 4 replicated pots were collected from each of the 5 AMF inoculum treatments and stored at 4°C until processing.

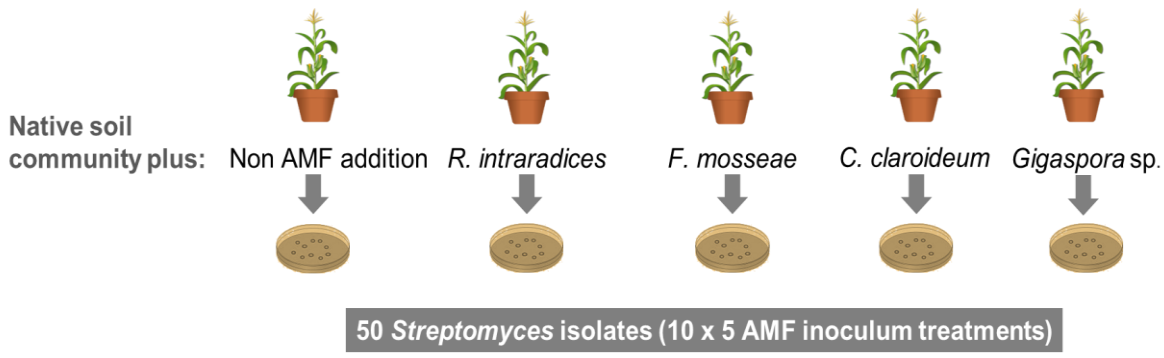

Figure 2. *Streptomyces* isolates were isolated from rhizosphere soil of 5 maize plant treatments of a pot experiment: non AMF addition or addition of one AMF species, *Rhizoglomus intraradices*, *Funneliformis mosseae*, *Claroideoglomus claroideum* or *Gigaspora* sp.

In order to obtain *Streptomyces* isolates, 5 g of rhizosphere soil sample from each of 4 replicates from each AMF inoculum treatment (given a total of 20 samples) were

weighed, added into an Erlenmeyer flask with 45 ml of saline solution 0.85% and shaken at 28°C for 30 min. Soil suspensions were dilution-plated on Emerson agar (Emerson 1941) (4 g l^{-1} beef extract, 1 g l^{-1} yeast extract, 4 g l^{-1} peptic digest of animal tissue, 10 g l^{-1} glucose, 2.5 g l^{-1} sodium chloride, 20 g l^{-1} agar, pH 7.0 ± 0.2) supplemented with 0.01% cycloheximide, 0.6% potassium dichromate and 0.6% nalidixic acid (to inhibit fungi and fast growing bacteria) (Zhang *et al*., 2006). Plates were incubated at 28°C for 120 h. For each AMF inoculum treatment, colonies that exhibited typical morphological characteristics of genus *Streptomyces* were subcultured and purified on ISP-2 agar (10 g l^{-1} malt extract, 4 g l^{-1} yeast extract, 4 g l^{-1} glucose, 20 g l^{-1} agar, pH 7.2 ± 0.2) (Shirling and Gottlieb, 1966). A total of 50 strains (10 from each AMF inoculum treatments) were chosen for further analysis.

2.2 – Antagonism assays of *Streptomyces* strains

2.2.1 – Antagonistic potential in monoculture

Inhibitory activity of the 50 *Streptomyces* strains was tested against 3 target bacteria, one common soil PGPR, *Bacillus megaterium* which is a Gram positive bacteria, and two important phytopathogenic bacteria, *Clavibacter michiganensis* and *Pseudomonas syringae*, which are Gram positive and Gram negative, respectively. These target bacterial strains were supplied by AMC Chemical, S.L. & Trichodex, S.A.

To study antagonistic potential in monoculture, the method described by Jauri and Kinkel (2014) was followed, with some modifications. Each *Streptomyces* strain was inoculated with a calibrated loopful (1 µl) on ISP-2 agar plates (4 replicates). Plates were incubated at 28°C for 96 h, and were then overlaid with one of the 3 target bacteria. For this step, *B. megaterium*, C. *michiganensis* and P. *syringae* were grown for 24, 72 or 48 h, respectively, in nutrient broth at 28°C, 160 rpm. Bacterial cultures were adjusted to an $OD_{600\,nm}$ of 0.5 and 1 ml was diluted in 9 ml of semi-solid nutrient agar (nutrient broth with 0.8% agar) to overlay the plate (approximately 10^6 cfu ml^{-1}). Plates were incubated for more 24 h at 28°C, and the radius of growth inhibition zones was measured in millimeters from the edge of the *Streptomyces* colony to the edge of the cleared zone (Figure 3A). *Streptomyces* strains were considered inhibitors when the radius of inhibition zones for at least one target bacteria was higher than 1 mm.

Antagonistic potential evaluation in monoculture consisted on 150 monoculture assays (50 isolates x 3 target bacteria) with 4 replicates.

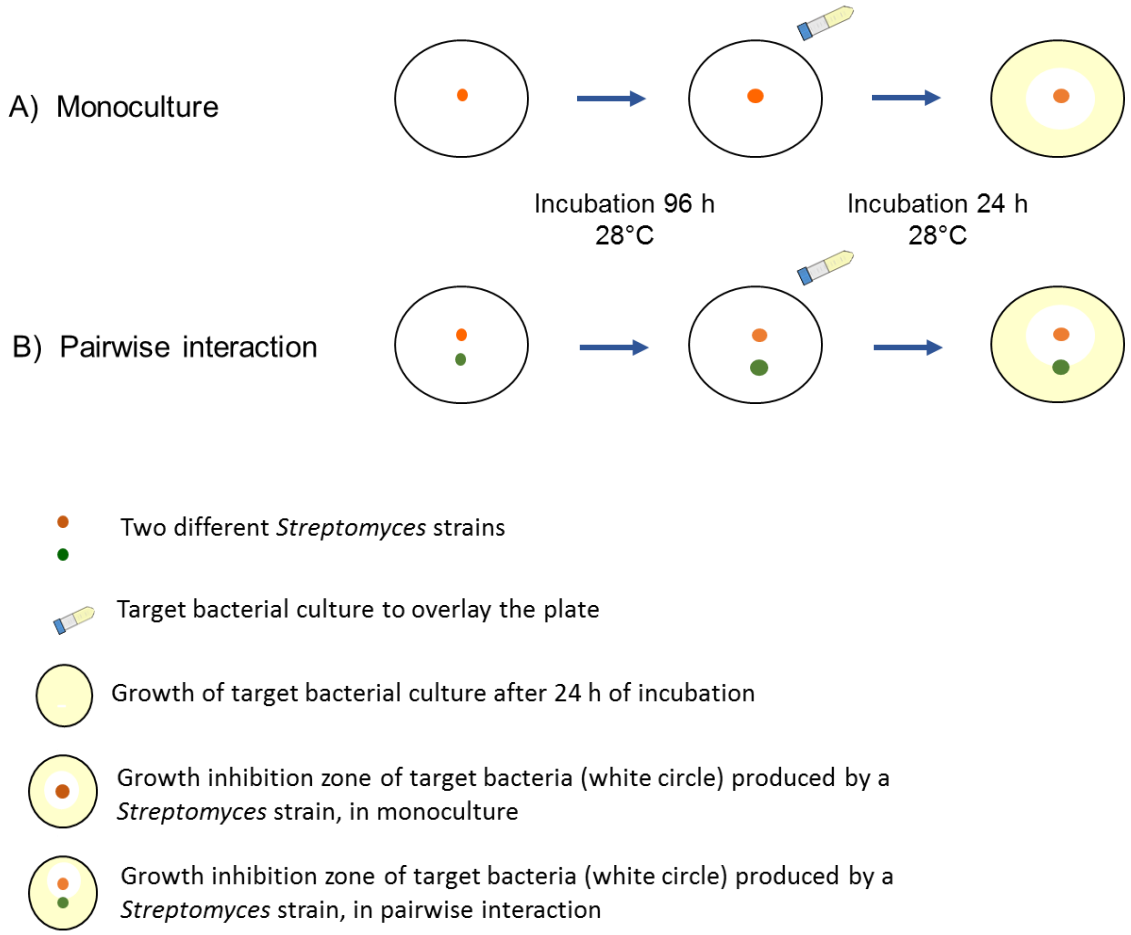

Figure 3. Schematic representation of antagonistic activity assay (A) in monoculture and (B) in *Streptomyces* pairwise interaction.

2.2.2 – Antagonistic potential in pairwise interactions

For pairwise interaction assays, 5 *Streptomyces* strains per each of the 5 mycorrhizosphere origins were randomly chosen from the strains that were used in the monoculture assays, given a total of 25 strains. Two types of interactions were tested: 1) interactions **within** mycorrhizosphere origins, *i. e.*, among pairs that were isolated from rhizosphere soil of the same AMF inoculum treatment; and 2) interactions **across** mycorrhizosphere origins, *i. e.*, among pairs isolated from rhizosphere soil of different AMF inoculum treatments. For the first type (within), in each treatment all possible

pairwise interactions were performed (10 per origin), thus, a total of 50 *Streptomyces* strain pairwise combinations was considered (10 x 5 origins). For the second type (across), 50 interactions were also performed. In this case, each *Streptomyces* strain from one AMF inoculum treatment interacted with only one other strain from each one of the others 4 AMF inoculum treatments.

For antagonistic activity in pairwise interaction assays, the method described by Jauri and Kinkel (2014) was followed, with some modifications. Each *Streptomyces* strain was inoculated 0.5 cm apart on ISP-2 agar plates (4 replicates) as in Figure 3B. The subsequent steps were carried out following the protocol previously described in section 2.2.1. For each pair, the highest growth inhibition zone was measured. The evaluation of antagonistic potential in pairwise interactions consisted of 300 pairwise interaction assays [(50 **within** + 50 **across** mycorrhizosphere origins) x 3 target bacteria)] with 4 replicates.

Type of changes in inhibitory activity was assessed by comparing the inhibition zones in monoculture assays with the one in the pairwise interaction (Figure 4) and defined as increases or decreases of inhibitory activity if the growth inhibition zone in the pairwise interaction was significantly higher or smaller than the highest (between the pair) in monoculture, respectively. Increase in inhibitory activity was evaluated as **intensification** when one isolate had the capacity to inhibit a bacterial target in monoculture, but this capacity was enhanced in the presence of a partner, and "**new" inhibition** when inhibitory activity was only observed when in pairwise interaction. Decrease in inhibitory activity was evaluated as **attenuation** if the inhibition zone of a target by a pair of strains was significantly lower than the highest inhibition zone when the strains were in monoculture, and **suppression** when one strain had the capacity to inhibit a bacterial target in monoculture, but it was lost in pairwise interaction.

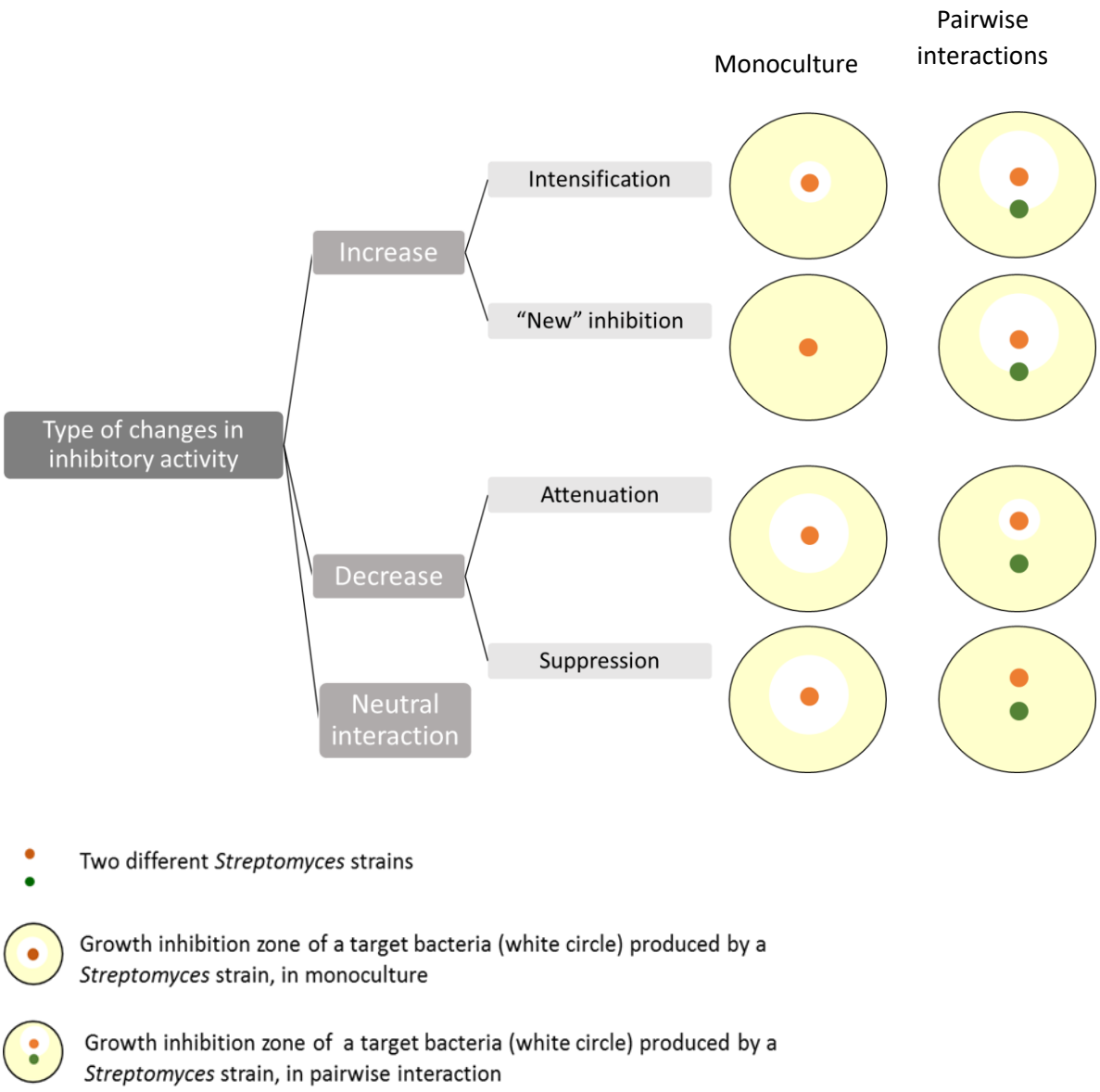

Figure 4. Schematic representation of the possible type of changes in *Streptomyces* strains inhibitory phenotype when in pairwise interactions.

2.3 - Molecular identification and genetic distance among *Streptomyces* strains

2.3.1 – DNA extraction

DNA was extracted following the method described by Pitcher *et al*. (1989), with some modifications. The DNA of each of the 25 strains involved in pairwise interaction assays was extracted from cells cultured on ISP-2 agar. The biomass was harvested to tubes containing 250 µl of lysis buffer (50 mM Tris; 250 mM NaCl; 50 mM EDTA; 0.3% SDS; 0.5% sarkosyl; pH 8.0) and glass beads were added corresponding to a

volume of 100 µl. Tubes were vortexed for 2 min, heated to 65°C for 30 min and vortexed again for additional 2 min. Then, 250 µl of GES buffer (5 M guanidium thiocyanate; 10 mM EDTA; 0.5% sarkosyl) were added, mixed by inversion, and tubes were kept on ice for 10 min. Subsequently, 125 µl of cold 10 M ammonium acetate were added and tubes were kept on ice for another 10 min followed by addition of 500 µl of chloroform:isoamyl alcohol (24:1) and mixed by inversion. The mixture was centrifuged at 14000 rpm for 10 min and the supernatant was collected to new tubes. DNA samples were precipitated by addition of 1 volume of 2-propanol, and collected by centrifugation at 14000 rpm for 10 min. The pellets were washed with cold 70% ethanol. After ethanol dried DNA were dissolved with 50 µl of TE (10 mM Tris-HCl; 1 mM EDTA; pH 8.0).

2.3.2 – PCR amplification of partial 16S rDNA gene

Partial 16S rDNA sequence (approximately 900 bp) was amplified using universal bacterial primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). PCR reactions were performed in a total volume of 50 µl containing 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM of dNTP's mixture, 50 pmol of each primer, 1 U of Taq DNA polymerase and 1 µl of extracted DNA were performed in a thermal cycler with the following settings: 94°C for 3 min, 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 3 min. To confirm the presence of expected size fragments, 5 µl of PCR products were analyzed by 1.2% agarose gel electrophoresis. Then, PCR products were purified using the JETQUICK PCR purification Spin kit (Genomed), and the existence of purified fragments was confirmed by 1.2% agarose gel electrophoresis. The PCR purified products were sent to BIOPREMIER, S.A. company for sequencing.

2.3.3 – Sequence analysis

For molecular identification of bacterial strains, partial 16S rDNA sequences were compared with sequences available in GenBank database of National Center for Biotechnology Information (NCBI) by using the Basic Alignment Search Tool (BLAST).

Sequence analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 6 software. Sequences were aligned by ClustalW algorithm and

used for calculation of genetic distance among *Streptomyces* strains using Tamura 3-parameter model.

2.4 – Statistical analysis

The effect of AMF inoculum treatment on the proportion of inhibitory *Streptomyces* strains was examined by a chi-square (χ^2) test.

A one-way analysis of variance (ANOVA) was used to test the influence of AMF inoculum treatment on the intensity of inhibition. Prior to ANOVA data were tested for homogeneity of variance and normal distribution to satisfy the assumptions of ANOVA.

A change in inhibitory phenotype induced by pairwise interaction was considered when a difference between mean inhibition zone produced by *Streptomyces* strains in monoculture and in pairwise interactions was significant according with a Student's *t-test*. Chi-square tests were used to examine differences in the proportion of changes in inhibitory activity between strain pairs from the same and different mycorrhizosphere origins, and to test for the effect of AMF inoculum treatment on the proportion of changes.

Student's *t-test*s were used to analyze significant differences in genetic distance among strains that changed or not their inhibitory capacity. To analyze if genetic distance among *Streptomyces* strains that resulted in changes in antimicrobial activity varied with mycorrhizosphere origin and with type of changes in inhibitory phenotypes during pairwise interactions a two-way ANOVA was performed. Comparison of frequency distributions of values of genetic distance (using unbinned data) between strain pairs from the same and different mycorrhizosphere origins was assessed using Kolmogorov-Smirnov goodness-of-fit test.

A probability of $P \leq 0.05$ was considered as representing a significance difference for all statistical tests used in this study.

All statistical analyses referred in this section were performed using IBM SPSS Statistics 21 software.

3 – Results

3.1 – The influence of mycorrhizal community on *Streptomyces* spp. antimicrobial activity in monoculture

The influence of rhizosphere-associated AMF community on the selection of *Streptomyces* antagonistic phenotypes was assessed by determining the proportion and the intensity of inhibition (antagonistic potential) of *Streptomyces* strains for each AMF inoculum treatment that inhibited the tested target bacteria *B. megaterium*, *C. michiganensis* and *P. syringae*.

Of the 50 strains, 24 (48%) showed the capacity to inhibit the growth of at least 1 of the target bacteria with only 8 (16%) inhibiting the 3 target bacteria tested (Figure 5). Curiously, the proportion of isolates that inhibited all bacterial targets is quite similar to the proportion that inhibited only 1 or 2 targets.

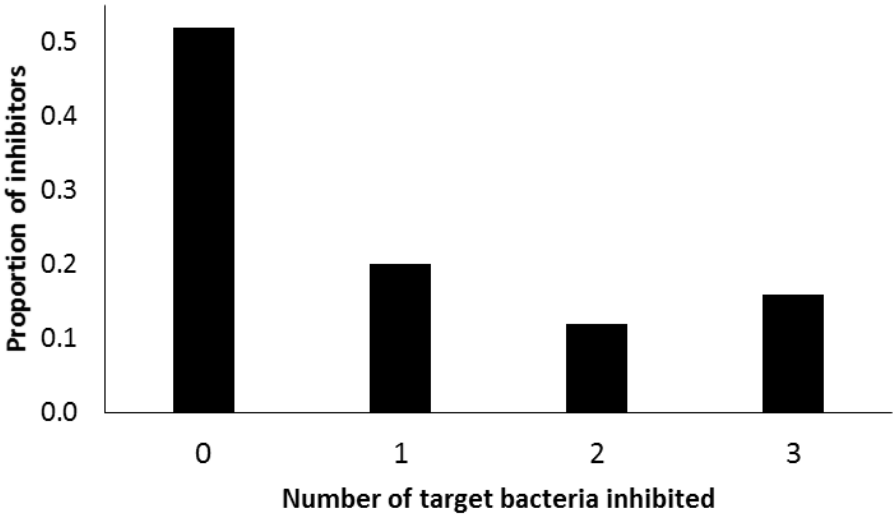

Figure 5. Proportion of *Streptomyces* strains in a total of 50 strains (10 in each of the 5 AMF inoculum treatments) that inhibited 0, 1, 2 or 3 of the tested target bacteria.

The AMF inoculum treatment had no significant effect on the proportion of *Streptomyces* strains that inhibited at least 1 bacterial target (Table 1, $\chi^2_{(4, 50)} = 1.122$, $P = 0.891$), nor on any other categories presented in Table 1 (χ^2, $P > 0.05$).

Table 1. Proportion of *Streptomyces* strains (in total of 50 strains) from each AMF inoculum treatment (n = 10 isolates per treatment) that inhibited the tested target bacteria.

AMF inoculum treatment	**At least 1 target inhibited**	**At least 2 targets inhibited**	**3 targets inhibited**	***B. megaterium* inhibited**	***C. michiganensis* inhibited**	***P. syringae* inhibited**	**Gram + and Gram - inhibited**
Non AMF addition	0.5	0.3	0.1	0.4	0.4	0.1	0.1
R. intraradices	0.4	0.2	0.2	0.2	0.4	0.2	0.2
F. mosseae	0.6	0.5	0.3	0.5	0.5	0.4	0.3
C. claroideum	0.5	0.1	0.0	0.3	0.2	0.1	0.0
Gigaspora sp.	0.4	0.3	0.2	0.3	0.4	0.2	0.2

Nevertheless, the proportion of inhibitor *Streptomyces* strains tended to be higher for *F. mosseae* inoculum addition. Of all strains, 14 (28%) had the ability to inhibit at least 2 target bacteria but only 8 had antimicrobial activity for both Gram positive and Gram negative targets, which suggests that the inhibitory effect of majority isolates is specific depending on the target bacteria. Overall, the Gram negative target bacterium (*P. syringae*) was the least inhibited.

The intensity of inhibition of tested bacteria by *Streptomyces* strains did not vary among AMF inoculum treatments from where *Streptomyces* strains were isolated (Figure 6; ANOVA $F_{(4,19)} = 0.428$, $P = 0.787$). In fact, a wide variability was observed in the intensity of inhibition among *Streptomyces* strains from each treatment.

Therefore, differences in AMF community composition (through distinct AMF inoculum addition) in the rhizosphere of a host plant species did not influence neither the frequency of *Streptomyces* antagonistic phenotypes nor its intensity of inhibition.

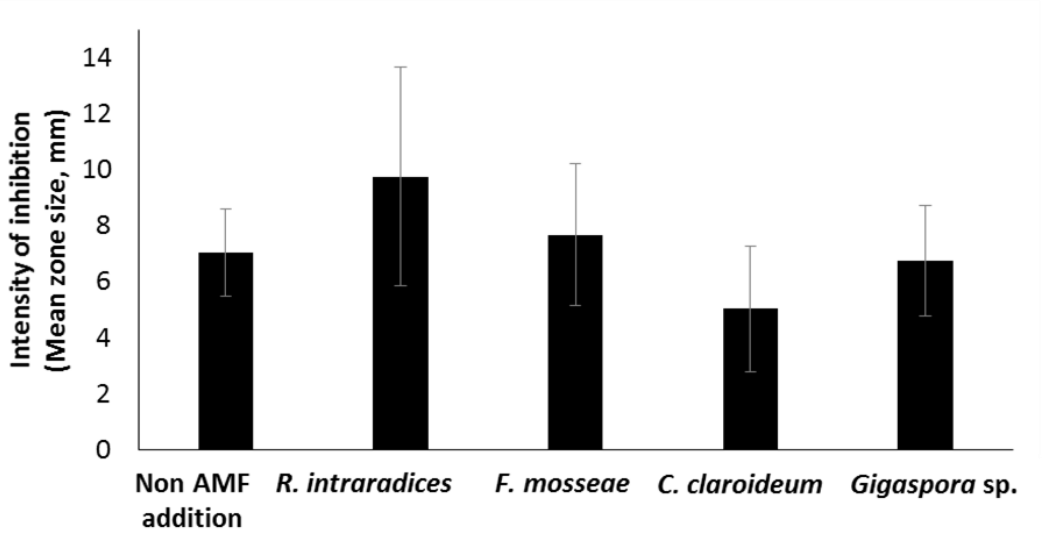

Figure 6. Intensity of inhibition among inhibitor *Streptomyces* strains from each AMF inoculum treatment. Bars are means ± SEM (n = 4–6; see table 1).

3.2 – Antagonistic phenotype changes induced by *Streptomyces* spp. pairwise interactions

The coexistence of microorganisms in nature promotes competitive and cooperative interactions that may influence the production of antimicrobial compounds. In this context, this study was based on a co-culture approach to induce the production of these bioactive molecules. Thus, 100 pairwise combinations among 25 *Streptomyces* strains were performed and antimicrobial activity was tested against the 3 target bacteria previously mentioned. Of 300 interaction cases tested, 117 (39%) showed changes in inhibitory activity of a target when compared to inhibition in monoculture. Those changes were both increases and decreases in inhibitory activity (20% and 19%, respectively). Among changes in antimicrobial activity, the results suggest a specificity in interaction since in several interactions involving the same partner, distinct outcomes were observed. For example, as shown in Figure 7A, the pairwise combination among strains MR1 and MR5 resulted in the so-called “new” inhibition (that is, none of strains inhibited *B. megaterium* in monoculture, but when in co-culture this target bacterium was inhibited), and the interaction among MR2 and MR5 lead to a total suppression of inhibitory activity while interaction among MR1 and MR2 did not show variation in inhibitory capacity. Moreover, in some interactions, the type of responses of antimicrobial activity during pairwise interaction was different depending on the target bacteria (examples in Figure 7A, B and C).

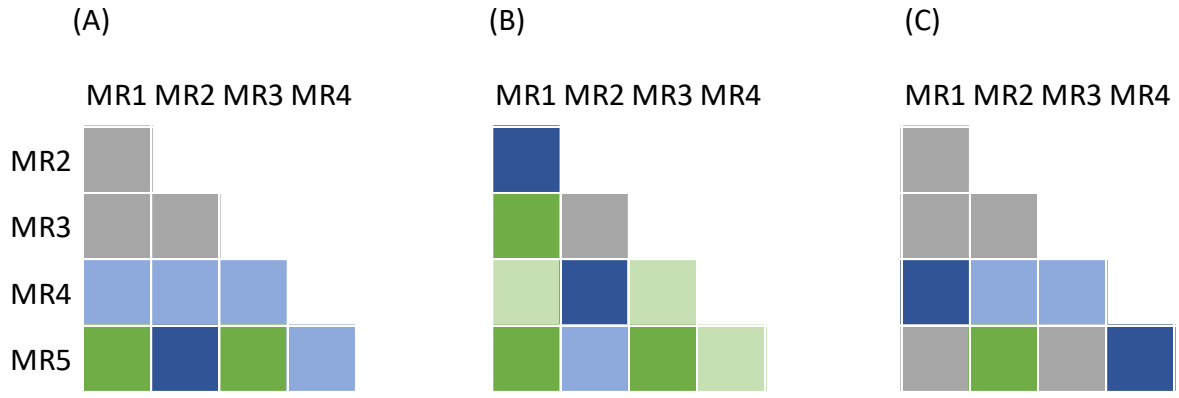

Figure 7. Examples of specificity on the responses of type of changes in inhibitory phenotype of some *Streptomyces* strains (MR1 – MR5) during pairwise interactions that resulted in intensification (light green), "new" inhibition (dark green), attenuation (light blue), suppression (dark blue) or did not change antimicrobial activity (gray) against the 3 target bacteria *B. megaterium* (A), *C. michiganensis* (B) and *P. syringae* (C).

3.2.1 – The influence of mycorrhizosphere origin on changes in antimicrobial activity

To evaluate the influence of rhizosphere mycorrhizal context on the selection for inhibitory phenotype-shifting interactions, the proportion and direction of changes in inhibitory activity in interactions among *Streptomyces* strains isolated from the same and different mycorrhizosphere origins (interactions **within** and **across** origins, respectively) were compared.

Considering all interactions, the proportion that resulted in changes in inhibitory activity was significantly higher in interactions within (45%) than across (33%) origins (Figure 8; $\chi^2_{(1,\ 300)} = 4.050$, $P = 0.044$). This suggests that there is a selection for *Streptomyces* strains that modulate its capacity to produce antimicrobial compounds in response to strains under the same selective pressure (influenced by AMF community).

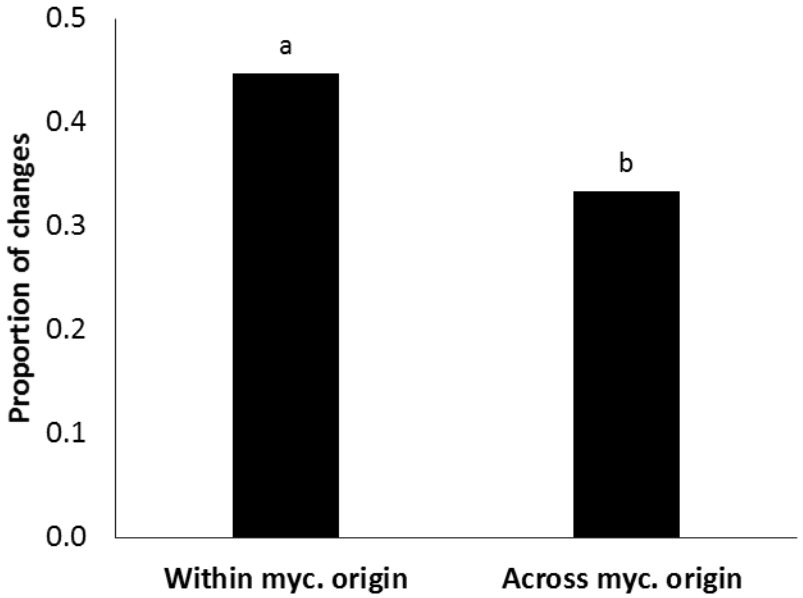

Figure 8. Proportion of changes in inhibitory activity in *Streptomyces* pairwise interactions within or across mycorrhizosphere origins, considering all pairwise interaction cases. Different letters indicate significant differences among origins (χ^2, $P \leq 0.05$).

In all pairwise interactions within the same mycorrhizosphere origin, there were 25% changes with increase in inhibitory activity, whereas only 15% increases were observed in all the pairwise interactions across origins (Figure 9; $\chi^2_{(1, 300)} = 4.083$, $P = 0.043$). This result is due to the proportion of interactions that resulted in the "new" inhibitions which were more than twice as high as observed in interactions across mycorrhizosphere origins (13% *vs*. 5%) (Figure 9; $\chi^2_{(1, 300)} = 6.064$, $P = 0.014$).

Interactions within the same mycorrhizosphere origin resulted mainly in changes that increased inhibitory activity, and on the contrary, interactions across mycorrhizosphere origins resulted in more decreases than increases. No significant differences in intensifications, attenuations and suppressions between within and across mycorrhizosphere origins were observed (χ^2, $P > 0.05$).

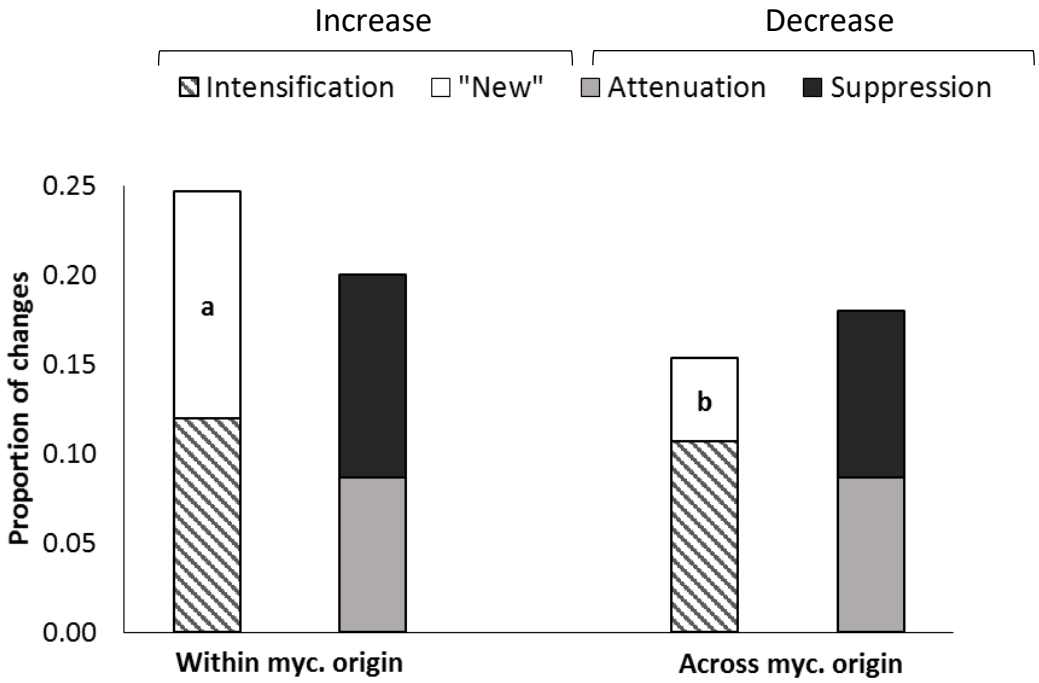

Figure 9. Type of changes in inhibitory phenotype of *Streptomyces* strains induced by pairwise interactions. Proportion of changes that produce increase, "new" inhibition, attenuation and suppression of antimicrobial activity in interactions within and across mycorrhizosphere origins in a total of 300 pairwise cases. Different letters indicate significant differences in "new" inhibitions comparing within and across mycorrhizosphere origins (χ^2, $P \leq 0.05$).

Overall, these results highlight the importance of mycorrhizosphere community context on the selection for *Streptomyces* strains that change its antimicrobial activity, especially to produce "**new" inhibitions**, which show that the use of co-culture approach integrating the ecological knowledge may be an asset to discover new antimicrobial compounds.

3.2.2 – The influence of mycorrhizal community on changes in antimicrobial activity

To evaluate whether mycorrhizal community composition associated with the rhizosphere influence the selection for inhibitory phenotype-shifting interactions, it was considered interactions among *Streptomyces* strains within the same mycorrhizosphere origin.

The total of changes in inhibitory activity was significantly different between AMF inoculum treatments (Table 2; $\chi^2_{(4, 150)} = 11.221$, $P = 0.024$). *Streptomyces* strains from the Non AMF addition treatment were more likely to alter their antagonistic phenotype

in co-culture (70%) than strains from rhizosphere soils where *R. intraradices* (30%) or *Gigaspora* sp. (37%) inoculum was added.

Curiously, decreases in inhibitory activity accounted for most of the changes occurred in the Non AMF addition treatment, while when AMF species inocula were introduced to soil, *Streptomyces* strains exhibited more increases (except for *Gigaspora* sp. soil addition; Table 2). Furthermore, the proportion of "new" inhibitions and attenuations of inhibitory activity was significantly influenced by AMF inoculum treatment ($\chi^2_{(4,150)}$ = 9.281, P = 0.050 and $\chi^2_{(4, 150)}$ = 12.297, P = 0.015, respectively). "New" inhibitions in antimicrobial activity were observed for all AMF inoculum treatments except for *Gigaspora* sp. soil addition, with higher proportion observed for *C. claroideum*, although only significant when compared with *Gigaspora* sp. treatment.

Interestingly, all interactions among strains from *C. claroideum* inoculum treatment showed extreme phenotypes since changes in inhibition were either "new" inhibitions or suppressions of antimicrobial activity.

Table 2. Proportion of total of changes in inhibitory activity by increase (intensification or "new" inhibition) and decrease (attenuation or suppression) for each AMF inoculum treatment. Different letters indicate significant differences among treatments (χ^2 P ≤ 0.05) (n = 30 pairwise interaction for each treatment).

AMF inoculum treatment	**Total of changes**	Increase		Decrease	
		Intensification	**"New"**	**Attenuation**	**Suppression**
Non AMF addition	0.70 [a]	0.10	0.20 [ab]	0.23 [a]	0.17
R. intraradices	0.30 [b]	0.17	0.10 [ab]	0.03 [ab]	0.00
F. mosseae	0.43 [ab]	0.17	0.10 [ab]	0.10 [ab]	0.07
C. claroideum	0.43 [ab]	0.00	0.23 [a]	0.00 [b]	0.20
Gigaspora sp.	0.37 [b]	0.17	0.00 [b]	0.07 [ab]	0.13

Results presented in Table 2 suggest that there are distinct dynamics among *Streptomyces* spp. in each AMF inoculum treatment leading to different outcomes in antimicrobial activity in interactions among *Streptomyces* spp.

3.3 – Relationship between genetic relatedness and changes in *Streptomyces* spp. inhibitory activity

Partial 16S rDNA sequence of the 25 strains involved in the interaction assays were compared with sequences available in GenBank database and isolates were identified by comparing to the closest bacterial relative (Table 3). This analysis confirmed that all strains belong to the genus *Streptomyces*.

Table 3. Identification of isolates of each AMF inoculum treatment, based on partial 16S rDNA similarity

AMF inoculum treatment	Isolates	Species	% identity
Non-AMF addition	MR1	*Streptomyces macrospureus*	99%
Non-AMF addition	MR2	*Streptomyces* sp.	99%
Non-AMF addition	MR3	*Streptomyces* sp.	99%
Non-AMF addition	MR4	*Streptomyces* sp.	99%
Non-AMF addition	MR5	*Streptomyces* sp.	99%
R. intraradices	MR6	*Streptomyces macrospureus*	99%
R. intraradices	MR7	*Streptomyces fradiae*	99%
R. intraradices	MR8	*Streptomyces* sp.	100%
R. intraradices	MR9	*Streptomyces luteogriseus*	99%
R. intraradices	MR10	*Streptomyces* sp.	100%
F. mosseae	MR11	*Streptomyces youssoufiensis*	100%
F. mosseae	MR12	*Streptomyces* sp.	100%
F. mosseae	MR13	*Streptomyces hygroscopicus*	99%
F. mosseae	MR14	*Streptomyces* sp.	99%
F. mosseae	MR15	*Streptomyces* sp.	99%
C. claroideum	MR16	*Streptomyces rutgersensis*	100%
C. claroideum	MR17	*Streptomyces fradiae*	100%
C. claroideum	MR18	*Streptomyces* sp.	100%
C. claroideum	MR19	*Streptomyces* sp.	99%
C. claroideum	MR20	*Streptomyces* sp.	100%
Gigaspora sp.	MR21	*Streptomyces arenae*	100%
Gigaspora sp.	MR22	*Streptomyces* sp.	99%
Gigaspora sp.	MR23	*Streptomyces* sp.	99%
Gigaspora sp.	MR24	*Streptomyces* sp.	99%
Gigaspora sp.	MR25	*Streptomyces* sp.	99%

To evaluate if changes in inhibitory phenotypes are dependent on genetic relatedness among strains in pairwise interactions, genetic distance among strains that changed or did not change their inhibitory activity (neutral interactions) was compared. Among all *Streptomyces* strain interactions, it was observed that changes in inhibitory activity occurred between more closely related individuals (Figure 10A; *t-test* T = 6.387, P = 0.027). Similar trend was observed for interactions within the same mycorrhizosphere origin (Figure 10B; *t-test* T = 2.587, P = 0.063), but not when strains were from different origins (Figure 10C; *t-test* T = 0.740, P = 0.503).

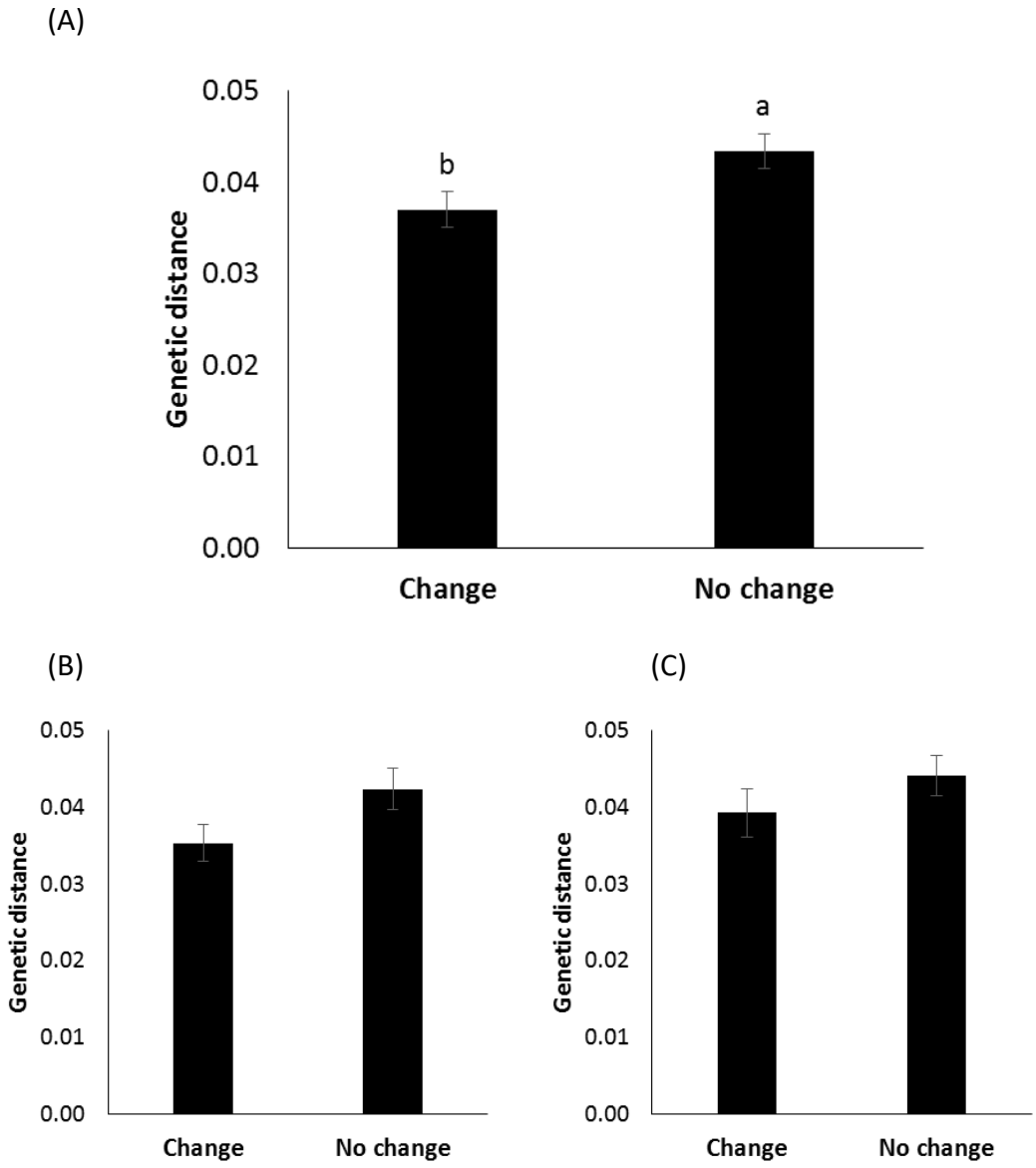

Figure 10. Genetic distance among all strain pairs (A), among strains from the same (B) and from different (C) mycorrhizosphere origins, that changed or not their antagonistic phenotype during *Streptomyces*

pairwise interactions. Bars are means ± SEM (n = 300 for A and n = 150 for B and C). Different letters indicate significant differences (*t-test*, P ≤ 0.05).

Considering only the cases that resulted in changes in the inhibitory activity, there were no significant differences in genetic distance between *Streptomyces* strain pairs from the same and different mycorrhizosphere origins, nor between type of changes during *Streptomyces* strain interactions (Figure 11; two-way ANOVA $F_{(1,109)}$ = 2.115, P = 0.149 for origin factor, $F_{(3,109)}$ = 1.921, P = 0.130 for type of changes factor, and $F_{(3,109)}$ = 0.285, P = 0.836 for origin x type interaction).

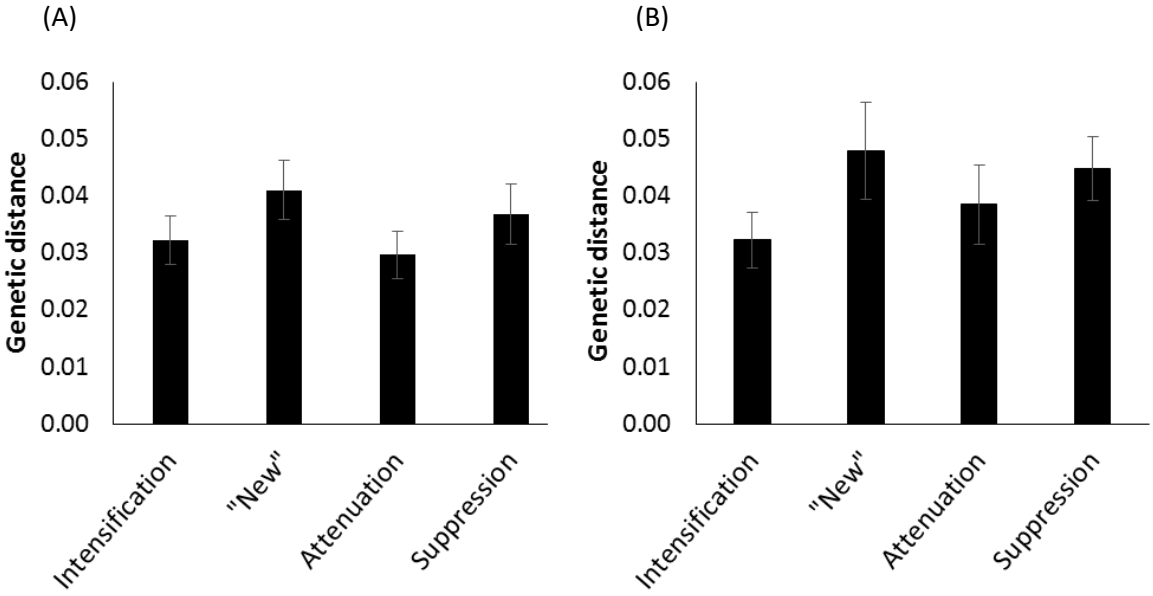

Figure 11. Genetic distance among *Streptomyces* strain pairs from the same (A) and different (B) mycorrhizosphere origins that resulted in changes of antimicrobial activity of *Streptomyces* strains. Direction of changes are indicated by increase (intensification or "new" inhibition) or decrease (attenuation or suppression) of inhibitory activity. Bars are means ± SEM (n = 7-19).

Since there were more changes in antimicrobial activity among more closely related strains (Figure 10A), the distributions of genetic distance values of strain pairs from the same and different mycorrhizosphere origin were compared to assess if there were more closely related pairs from the same origin. However, the shape of the frequency distributions did not differ between origins (Figure 12; Kolmogorov-Smirnov test, P = 0.544).

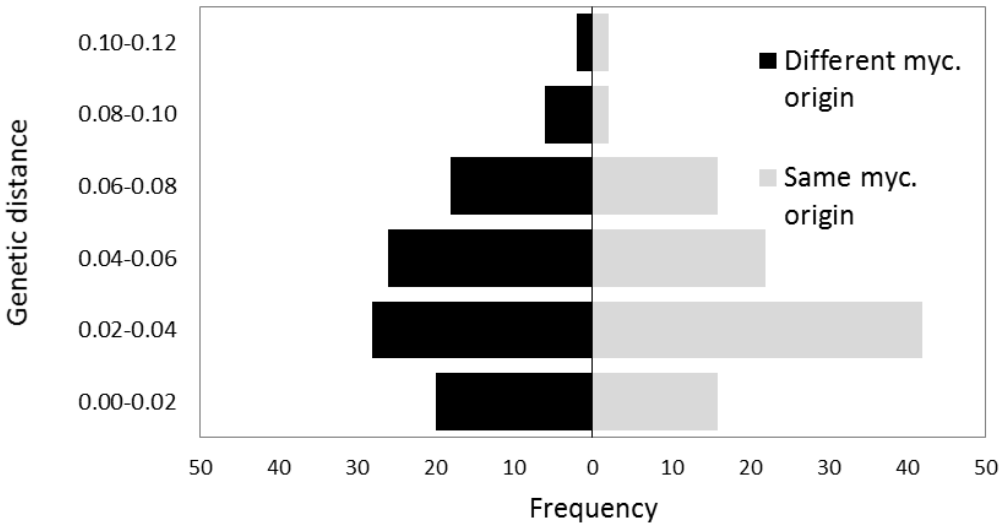

Figure 12. Frequency distribution of genetic distance among *Streptomyces* strains from the same or different mycorrhizosphere origins.

These results suggested that although genetic relatedness seems to be an important determinant to change inhibitory activity of *Streptomyces* strains in co-culture, the frequency of changes in interactions within the same and across different mycorrhizosphere origins was not dependent on the genetic relatedness among the *Streptomyces* strains.

4 – Discussion

There has been increasing interest in studying and exploring social interactions among microorganisms for understanding microbial community dynamics and for novel drug discovery. It has been shown that the composition of the interacting microbes influences the antagonistic activity and the production of antimicrobial compounds (Traxler *et al*., 2013; Tyc *et al*., 2014). In soils, existing data suggest that biotic factors such as plant community characteristics and the identity of plant species can impact the frequency of antagonistic phenotypes and the diversity of antimicrobial compounds (Bakker *et al*., 2010, 2013a). The present study provides evidence that the biotic (mycorrhizal) context of the rhizosphere is an important factor influencing the outcome of *Streptomyces* spp. interactions in antimicrobial activity.

Rhizosphere is one of the most dynamic natural environments harboring a vast biodiversity with uncountable inter-kingdom interactions. The association between plant roots and mycorrhizal fungi plays a pivotal role on the composition of rhizosphere microbiota (Rillig *et al*., 2006), since microorganisms feed on root exudates which are enriched in nutrients such as sugars, amino acids and organic acids (Philippot *et al*., 2013). The root exudation patterns are influenced by many factors including the AMF species in symbiosis with plants (Johansson *et al*., 2004). Thus, both the plant and associated AMF shape the microbial community structure in the rhizosphere. The present study follows a previous work showing that microbial composition of AMF and bacterial communities (assessed as cultivation-dependent bacterial density and as 454 pyrosequencing) in maize plant rhizosphere was differently affected by the identity of AMF species inoculum added (Cruz *et al*., data not published). Taken into account these previous results, in the present study it was evaluated whether the different AMF inocula addition (leading to different rhizosphere AMF communities) could lead to variation in the antagonistic potential of bacteria from the genus *Streptomyces*. Among 50 *Streptomyces* strains isolated, 48% showed the capacity to inhibit the growth of at least one of the 3 target bacteria. However, neither the proportion of inhibitory *Streptomyces* strains nor its strength of inhibition (intensity of inhibition) differed across AMF inoculum treatments. These findings are not in line with some works that have observed that both frequency of inhibitors and inhibitory intensity could vary if the

environments where *Streptomyces* spp. live were more or less competitive, for instance by niche overlap among coexisting individuals (Davelos *et al*., 2004a; Kinkel *et al*. 2014). While there was no evidence that distinct AMF communities colonizing the rhizosphere of a plant species influence the selection of antagonistic phenotypes of *Streptomyces* spp., it cannot completely be ruled out a possible AMF effect on the assembling of antagonistic phenotypes because the number of isolate samples (10 per AMF treatment) might have been too small to detect any significant differences or because antagonism is generally socially-mediated (Abrudan *et al*., 2015). The latter explanation may be applied since those results were observed for strains in monoculture, and it has been documented that *Streptomyces* strains often modulate their antimicrobial activity in response to metabolic by-products released by neighbor strains (Antoraz *et al*., 2015). In fact, in the present study it was found that the antimicrobial activity during *Streptomyces* spp. interactions is commonly altered. Among all interactions tested, 39% showed changes in inhibitory phenotypes of at least one co-culture "player". The changes were both increases and decreases in the inhibitory capacity. These data showing an high frequency of changes in inhibitory phenotype of *Streptomyces* strains when in the presence of a coexisting partner are in accordance with recently observed findings in pairwise interactions with other natural soil *Streptomyces* communities (Jauri and Kinkel, 2014; Abrudan *et al*., 2015).

Results of the present study also support that the co-culture is a very useful technique to induce the production of antimicrobial compounds. As observed in previous works (Jauri and Kinkel, 2014; Abrudan *et al*., 2015), the type of response in pairwise interactions (increase or decrease in inhibitory capacity) was specific dependent on the partner identity. Moreover, the use of three target bacteria which may vary in their susceptibility to antimicrobial compounds allowed to achieve a more sensitive method to detect inhibitory phenotype-shifting interactions. These results suggest that signal communication between members of this group of bacteria is an advantage to optimize fitness benefits, with individuals modifying the antagonistic phenotype according to their biological context (Cornforth and Foster, 2013).

Abrudan *et al*. (2015) defended that both increase and decrease in antimicrobial activity presumably benefit *Streptomyces* species in a social competitive environment, and that microorganisms respond to the presence of competitors by producing

antibiotics to kill them, or otherwise by suppressing the antibiotic production in order to increase their chance to survive. Although this study was not aimed at evaluating if changes in antimicrobial activity during *Streptomyces* strain co-culture were result of cooperative or competitive interactions, it is very likely that both have occurred. While decreases in inhibitory activity of a partner most likely resulted from competitive interactions, for instance through the production of antibiotic-degrading enzymes or by interfering with the signals which regulate its production (Wright, 2005) increases in the inhibitory activity may have resulted from competitive or cooperative interactions. Cooperation may have occurred through cooperative signals released by a partner which induce a response leading the other partner to increase their inhibitory capacity, and thus eliminate a competitor of both individuals. Competition may have occurred when one strain perceive a competitor and respond by intensifying the production or producing "new" antimicrobial compounds to kill the partner, thereby reducing direct threats. These response-sensing mechanisms to regulate antimicrobial activity may be useful to reduce costs associated with constitutive expression of antibiotic pathways (Cornforth and Foster, 2015). Moreover, this dichotomy between increase and decrease in the antimicrobial activity may be one explanation for the maintenance of microbial biodiversity in the soil since lethal interactions can be converted into neutral ones (Kelsic *et al*., 2015).

Integrating the ecological context of bacterial strains in studies for new drug discovery is of major importance (Smanski *et al*., 2016). Results of this work showed a higher proportion of changes in antagonistic phenotypes of *Streptomyces* strains in pairwise interactions when the strains were from the same mycorrhizosphere origin (45%) than when were isolated from different origins (33%). Specifically, when analyzing by the type of response, there were more increases in inhibitory activity, but most important, there were more interactions that resulted in the so-called "new" inhibitions, that is, two strains did not show inhibitory activity against a bacterial target in monoculture, but when they were both in co-culture such target was inhibited. The distinct inhibition profiles (type of changes) across AMF treatments suggest that the introduction of a single AMF inoculum into a native soil AMF background community alters the microbial community dynamics. Overall, the addition of AMF inoculum to the rhizosphere of maize plants decreased the frequency of *Streptomyces* strains

interactions that resulted in changes in antimicrobial activity. Furthermore, the frequency of "new" inhibitions varied with AMF inoculum added. All these data support the prediction that the frequency and type of changes in antimicrobial activity induced by pairwise interactions are mycorrhizosphere context dependent (origin and AMF community). Though plant species identity has been shown to have impacts on *Streptomyces* antagonistic potential (Bakker *et al*., 2013b), the present work indicates that variation in mycorrhizal fungal community associated with the rhizosphere of the same host plant is a key player influencing *Streptomyces* spp. interactions. More important than AMF identity, there is a clear potential in the use of *Streptomyces* strains that shared the same mycorrhizosphere community context in a co-culture approach to induce the production of antimicrobial compounds.

Genetic relatedness among bacterial strains may be an important factor in explaining changes in antimicrobial activity during pairwise interactions. Schoustra *et al*. (2012) showed that antagonistic interactions are unlikely to occur among too genetically distant strains because resource competition is stronger among closely related individuals than among too distant ones. In fact, this work showed that strains in which the inhibitory capacity of at least one of them was altered during pairwise interactions were more closely related than strains that produced neutral interactions. However, genetic distance among *Streptomyces* strains isolated from the same or different mycorrhizosphere origins was quite similar. Additionally, the different type of changes in antimicrobial activity were not genetic relatedness-dependent. The lack of relation between genetic relatedness and mycorrhizosphere-context dependence in inhibitory phenotype shifting suggests that response outcomes of social interactions among *Streptomyces* strains may be more dependent on other factors, such as the association between inhibitory and resistance antibiotic activities (Davelos *et al*., 2004b; Jauri and Kinkel, 2014), and nutrient overlap (Jauri and Kinkel, 2014; Kinkel *et al*., 2014).

5 – Conclusions

This study builds on previous recognition that microbial interactions can be biotic context dependent and bacteria of the genus *Streptomyces* often modulate their antimicrobial activity in response to a specific partner, to explore the influence of rhizosphere biotic conditions, namely plant-mycorrhizal associations, in the dynamics of *Streptomyces* spp. antimicrobial activity. Data of this study provides evidence that the frequency of changes in antimicrobial activity due to *Streptomyces* spp. interactions is higher when strains came from the same mycorrhizosphere origin, that is, from rhizosphere associated with the same mycorrhizal fungal community, highlighting that *Streptomyces* populations behave differently depending on the plant-mycorrhizal context from which they were originated.

The results of this work add an additional layer of complexity beyond microbe-microbe interactions when studying soil microbial dynamics. Results highlight the importance of plant-microbe associations (plant-AMF) in structuring microbial communities and on microbe-microbe interactions in the rhizosphere.

The finding that mycorrhizosphere origin of strains was an important factor in *Streptomyces* spp. interaction outcomes, specifically, in the production of “new” inhibitions during pairwise interactions, underscores that mycorrhizosphere is an important hotspot for the selection of potential partners for the co-culture approach aiming to boost antibiotic discovery.

Taking into consideration the huge bacterial diversity in a multitude of habitats and the countless possibilities of interactions that can be explored, the results of this work emphasize the advantage in linking the ecological knowledge and the microbial social context to studies for new drugs discovery, and thus, we should prioritize co-existing bacterial strains with greatest potential to elicit the production of new antimicrobial compounds.

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